Biochimica et Biophysica Acta, 945 (1988) 121–126
Elsevier

BBA 74189

Abnormal erythrocyte membrane cytoskeleton structure in chronic myelogenous leukaemia

Joyoti Basu [a], Manikuntala Kundu [a], Madan Mohan Rakshit [b] and Parul Chakrabarti [a]

[a] *Department of Chemistry, Bose Institute, Calcutta and* [b] *Haematology Department, NRS Medical College and Hospital, Calcutta (India)*

(Received 13 June 1988)

Key words: Leukemia, chronic myelogenous; Membrane skeleton; Heat sensitivity; (Human)

Chronic myelogenous leukaemia (CML) is a haematologic malignancy characterised by excessive growth of myeloid cells and their progenitors. Our studies show that there are several abnormalities in CML red blood cells. The proportion of spectrin dimers compared to tetramers extracted from membranes at 4°C, under low ionic strength conditions, increased in CML erythrocytes. These also displayed abnormal thermal sensitivity (between 45 and 46 instead of 49°C). Decreased spectrin tetramer formation observed in several hereditary anaemias has been associated with decreased red cell deformability leading to splenic sequestration. This could also be one of the causes of the severe anaemia observed in CML. Crosslinking with the bifunctional reagent, dimethyl adipimidate (8.6 Å) showed significant organizational modification of not only spectrin, but other cytoskeletal components such as ankyrin, bands 4.2 and 5. Enhanced concanavalin A agglutinability of CML erythrocytes also suggests altered topographic distribution of a functionally important membrane protein, band 3.

Introduction

The red cell membrane is composed of a lipid bilayer underlying which is a meshwork of peripheral proteins located at the cytoplasmic surface. This flexible and elastic meshwork of proteins comprises the cytoskeleton. It is responsible for maintaining the biconcave shape of the erythrocyte, for its reversible deformability and for membrane structural integrity [1,2]. Spectrin, a long, rod-like molecule of two non-identical subunits, is the major component of the membrane skeleton. Spectrin dimers associate into tetramers and higher oligomers. The tetramer is the predominant form of spectrin in the normal erythrocyte membrane [3]. Spectrin tetramers interact with protein 4.1 and actin to form the cytoskeleton network [4,5]. This network is linked to the membrane by two proteins. Protein 4.1 binds to the end of spectrin distal to its self-association site and attaches the spectrin-actin network to the membrane through glycophorin A [6]. Ankyrin binds to the β subunit of spectrin and attaches it to the membrane via band 3 [7]. The membrane skeleton restricts the lateral mobility of integral membrane proteins in the plane of the membrane [8] and also stabilizes the slow transbilayer movement of the phospholipids of the membrane [9,10]. Defects and de-

Abbreviations: CML, chronic myelogenous leukaemia; DMA, dimethyl adipimidate.

Correspondence: P. Chakrabarti, Department of Chemistry, Bose Institute, 93/1 Acharya Prafulla Chandra Road, Calcutta 700 009, India.

ficiencies in the skeletal components have been described in several hereditary haemolytic anaemias, where they give rise to mechanically and/or thermally unstable erythrocytes [11] with shortened life-spans. Chronic myelogenous leukaemia is a haematologic malignancy characterized by excessive growth of myeloid cells and their progenitors [12]. The present studies show that there are abnormalities in the erythrocyte membrane skeletal proteins which may affect the normal functions of the red blood cells and may account in part, for the severe anaemia associated with the disease.

Materials and Methods

Materials

Reagents for gel electrophoresis, dimethyl adipimidate (DMA), concanavalin A and glutaraldehyde (EM grade) were obtained from Sigma (St. Louis, MO, USA). Sepharose-4B was obtained from Pharmacia (Uppsala, Sweden).

Blood from leukaemic patients was obtained, in heparin, from NRS Medical College and Hospital, Calcutta. Blood was also collected from healthy volunteers. Cells were centrifuged at $1000 \times g$ for 5 min and washed repeatedly with 10 mM Tris-HCl (pH 7.4)/0.15 M NaCl (Buffer 1). After each centrifugation, a portion from the top of the cell pellet was discarded to ensure removal of leucocytes.

Preparation of ghosts

Erythrocytes were lysed in cold 10 mM Tris-HCl (pH 7.4). Membranes were sedimented at $22000 \times g$ for 15 min at 4°C and washed three or four times in the same buffer to free them from haemoglobin.

Extraction of membrane skeletal components

Membrane skeletal components were extracted according to Gratzer [13]. Briefly, ghosts in 1 mM Tris-HCl (pH 8.0)/0.1 mM EDTA/0.1 mM phenylmethylsulphonyl fluoride were dialysed against the same buffer for 20–24 h at 4°C. Membranes were sedimented and the supernatants were fractionated on a Sepharose-4B column equilibrated against the same buffer.

Heat treatment of erythrocytes

Erythrocytes (normal and leukaemic) were incubated at different temperatures (37, 42, 46 and 49°C) for 30 min and immediately cooled on ice.

Scanning electron microscopy

Heat-treated erythrocytes were sampled and fixed in 2.5% glutaraldehyde in phosphate-buffered saline for 30 min. They were then incubated in 1% osmium tetroxide in phosphate-buffered saline for 30 min, dehydrated with graded ethanol and finally suspended in isopropanol. Erythrocytes were air-dried on glass coverslips, coated with gold and examined in a scanning electron microscope (Model No. PSEM-500, Philips, The Netherlands).

Crosslinking with DMA

Ghosts were treated with the bifunctional crosslinking agent DMA, as described by Ji and Nicolson [14]. Ghosts in Buffer 1 were incubated with 0.1 mg/ml DMA for 60 min at room temperature. The reaction was terminated with 0.05 M ammonium acetate. The membranes were pelleted at $22000 \times g$ for 15 min and washed once in 10 M Tris-HCl (pH 7.4). The crosslinked ghosts were taken in 1% SDS and run on 7.5% SDS-polyacrylamide gels according to Laemmli [15].

Agglutination of cells with concanavalin A

A 0.4% (v/v) suspension of erythrocytes was mixed with an equal volume of concanavalin A of different concentrations in Buffer 1. Controls containing 50 mM methyl mannopyranoside were run side by side with concanavalin A. After incubation at 37°C for 60 min, the extent of agglutination was determined under the microscope.

Results and Discussion

Extraction and fractionation of skeletal components

The treatment of erythrocyte ghosts with low ionic strength buffer at 4°C for a prolonged period (24–48 h) and subsequent fractionation on Sepharose 4B leads to three major peaks attributable to the spectrin-actin-band 4.1 complex, tetrameric spectrin and dimeric spectrin [16]. In normal human erythrocytes, spectrin is predominantly in tetrameric form. Supernatants from CML ghosts treated similarly in low ionic strength showed

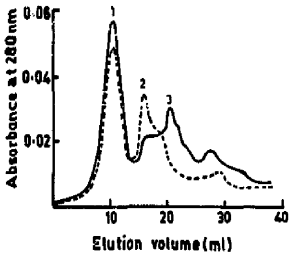

Fig. 1. Elution pattern of cytoskeletal proteins from normal and CML erythrocyte ghosts at 4°C, under low ionic strength conditions from a Sepharose-4B column. The broken line represents the elution from normal, and the continuous line represents the elution from CML erythrocyte ghosts. Peaks 1, 2 and 3 correspond to the spectrin-actin-band 4.1 complex, tetrameric spectrin and dimeric spectrin, respectively. Similar elution patterns have been observed in the case of five normal healthy donors and five CML patients.

considerable amounts of spectrin dimer formation and reduced tetramer (Fig. 1). That the tetramer is crucial for the stability of the membrane has been demonstrated in experiments in which the tetramer has been partially converted to dimers in situ on the ghost membrane. This conversion is accompanied by a considerable loss of mechanical stability [17]. Reduced proportion of spectrin tetramers has also been reported in several cases of hereditary anaemia [18–21].

Morphology of heated CML and normal erythrocytes

CML erythrocytes were found to be thermally more sensitive than normal erythrocytes (Fig. 2). At 46°C, CML cells became poikilocytes. Normal cells underwent the same transformation at 49°C. Similar observations have been made in patients with hereditary elliptocytosis, in which the proportion of spectrin dimer extracted from membranes at 4°C under low ionic strength conditions, increased between 25 and 56% (normal, 15 ± 2%) [22].

Crosslinking with DMA

Favourable arrangements of α and β chains at the head-end allow the formation of spectrin tetramers or oligomers, which are the functional units in the membrane skeleton. Similarly, actin, ankyrin, band 4.1 and the tail end of spectrin where it interacts with band 4.1, should be in a favourable position to build up the protein meshwork which is believed to be responsible for the shape and stability of the red cell. The arrangement of skeletal proteins in the CML erythrocyte ghost was studied using the bifunctional crosslinking agent DMA with a span of 8.6 Å. In DMA concentrations at which normal ghosts failed to show any crosslinking, CML ghosts showed a decrease in the amounts of band 1, 2, 2.1, 2.2, 2.3, 4.2 and 5, compared to untreated ghosts (Fig. 3), and the formation of high-molecular-weight masses which could not penetrate the gel. This suggests that the skeletal proteins in CML ghosts undergo a spatial redistribution so that they become crosslinkable by a bifunctional reagent with a span of 8.6 Å. It has previously been reported that spectrin becomes crosslinked via disulphide bonds in CML [23].

Agglutinability with concanavalin A

The effect of defective spectrin tetramer formation and reorganization of cytoskeletal components on the topographic distribution of other functionally important membrane proteins was studied by determining the agglutinability of CML erythrocytes using concanavalin A. CML eythrocytes were agglutinated by 2 μg/ml concanavalin A (Fig. 4), whereas normal erythrocytes are agglutinated by concanavalin A only after trypsin digestion. No significant increase in the number of concanavalin A receptors was found in CML compared to normal erythrocytes, by studying the binding of [^{3}H]concanavalin A to the CML red blood cells (data not shown). Moreover, increase in the number of lectin receptors is not likely to be of importance, since only a small fraction (5%) of total receptors participates in agglutination [24,25]. Extensive work with lectin-induced agglutination of transformed cells has shown that, after lectin binding, the receptor molecules must undergo aggregation in the membrane for agglutination to occur [26]. Normal cells do not show this receptor aggregation, although they bind concanavalin A. It has been suggested that the transmembrane lectin receptor (band 3) is anchored to the cytoskeletal structure and is incapable of lateral movement [27,28]. It has been reported that the lateral mobility of band 3 is restricted by interactions of

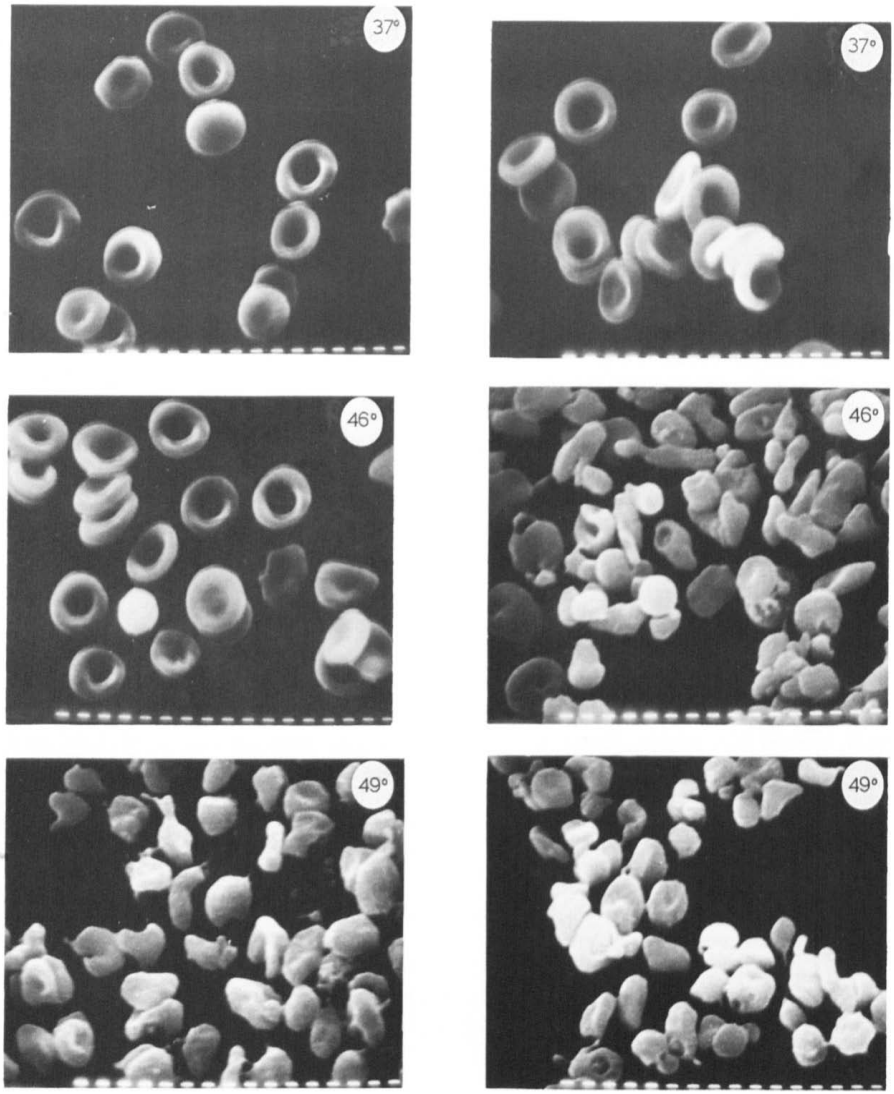

Fig. 2. Scanning electron micrographs of CML and normal erythrocytes, incubated at different temperatures, as described in Materials and Methods. Magnification: 2880×.

its cytosplasmic domain with the cytoskeletal network; it can pass the network when spectrins are in dissociated dimers, but cannot pass when they are in tetramers [29]. Therefore, a difference in cytoskeletal control of receptor mobility may be the major determinant of the enhanced concanavalin A-agglutinability of CML erythrocytes.

Membrane deformability, flexibility and morphology are primarily dependent on the control by the cytoskeletal network underlying the cyto-

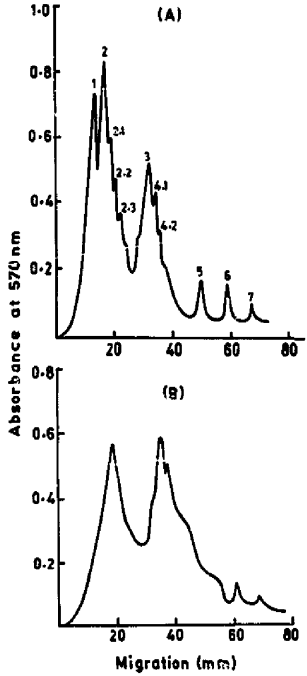

Fig. 3. Densitometric scanning of Coomassie blue-stained gels of CML erythrocyte ghosts. Ghosts were treated with DMA as described in Materials and Methods. DMA-treated and untreated ghosts were dissolved in 1% SDS and applied on a 7.5% polyacrylamide gel. Coomassie blue-stained-gels were scanned at 570 nm. (A) Untreated CML ghosts; (B) DMA-treated CML ghosts. Bands have been numbered according to the nomenclature of Fairbanks et al. [39]. Ghosts from five CML patients showed similar patterns before and after treatment with DMA.

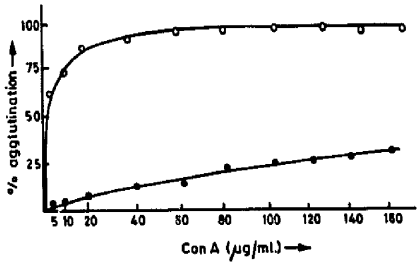

Fig. 4. Concanavalin A agglutination of normal (●——●) and CML (○——○) erythrocytes. Agglutination was carried out as described in Materials and Methods.

plasmic surface [11,30–34]. The physical state of skeletal proteins is important in these functions [35,36]. Reduced spectrin tetramer formation in CML may be one of the factors closely associated with the severe anaemia. Reorganisation of other cytoskeletal proteins such as ankyrins, band 4.2 and 5, and the possible altered topographic distribution and/or mobility of the functionally important protein, band 3, represent other significant red cell membrane abnormalities in chronic myelogenous leukemia.

The membrane skeleton plays a key role in maintaining the asymmetric distribution of phospholipids in the red blood cell membrane. Accelerated transbilayer movement of phospholipids has been observed in sickle-cell anaemia [37] and hereditary pyropoikilocytosis [38]. It will be of interest to see how transbilayer movement of phospholipids is affected in CML.

Acknowledgement

This work was supported by a grant from the Department of Science and Technology, Government of India.

References

1 Goodman, S.R. and Shiffer, K.A. (1983) Am. J. Physiol. 244, C121–C141.
2 Bennett, V. (1985) Annu. Rev. Biochem. 54, 273–304.
3 Ungewickell, E., Bennett, P.M., Calvert, R., Ohanian, V. and Gratzer, W.B. (1979) Nature 280, 811–814.
4 Fowler, V. and Taylor, D.L. (1980) J. Cell Biol. 85, 361–376.
5 Cohen, C.M., Tyler, J.M. and Branton, D. (1980) Cell 21, 875–883.
6 Anderson, R.A. and Lovrien, R.E. (1984) Nature 307, 655–658.
7 Weaver, D.C., Pasternack, G.R. and Marchesi, V.T. (1984) J. Biol. Chem. 259, 6170–6175.
8 Goodman, S.R. and Branton, D. (1978) J. Supramol. Struct. 8, 455–463.
9 Franck, P.F.H., Chiu, D.T.-Y., Op den Kamp, J.A.F., Lubin, B., Van Deenen, L.L.M. and Roelofsen, B. (1983) J. Biol. Chem. 258, 8435–8442.
10 Lubin, B., Chiu, D. Bastacky, B., Roelofsen, B. and Van Deenen, L.L.M. (1981) J. Clin. Invest. 67, 1643–1649.
11 Marchesi, V.T. (1985) Annu. Rev. Cell Biol. 1, 531–561.
12 Champlin, R.E. and Golde, D.W. (1985) Blood 65, 1039–1047.
13 Gratzer, W.B. (1982) Methods Enzymol. 85, 475–480.
14 Ji, T.H. and Nicholson, G.L. (1974) Proc. Natl. Acad. Sci. USA 71, 2212–2216.

15 Laemmli, U.K. (1970) Nature 227, 680–685.
16 Ungewickel, E. and Gratzer, W.B. (1978) Eur. J. Biochem. 88, 379–385.
17 Liu, S.C. and Palek, J. (1980) Nature 285, 586–588.
18 Evans, J.P., Baines, A.J., Hann, I.M., Al-Hakim, I., Knowles, S.M. and Hoffbrand, A.V. (1983) Br. J. Haematol. 54, 163–172.
19 Coetzer, T. and Zail, S.A. (1982) Blood 59, 900–995.
20 Burke, B.E. and Shotton, D.M. (1983) Br. J. Haematol. 54, 173–187.
21 Lux, S.E. (1979) Semin. Haematol. 16, 21–51.
22 Lecomte, M.C., Dhermy, D., Solis, C., Ester, A., Feo, C., Gautero, H., Bournier, O. and Boivin, P. (1985) Blood 65, 1208–1217.
23 Kumar, A. and Gupta, C.M. (1983) Nature 303, 632–633.
24 Schnebli, H.P. and Bachi, T. (1975) Exp. Cell Res. 91, 175–183.
25 Kabat, E.A. and Chen, S. (1985) J. Cell Biol. 101, 652–659.
26 Nicolson, G.L. (1976) Biochim. Biophys. Acta 43, 648–651.
27 Edelman, G.M. (1976) Science 192, 218–226.
28 Ash, J.F., Voigt, P.K. and Singer, S.J. (1976) Proc. Natl. Acad. Sci. USA 73, 3603–3607.
29 Tsuji, A. and Ohnishi, S. (1986) Biochemistry, 25, 6133–6139.
30 Mohandas, N., Chassis, J.A. and Shohet, S.B. (1983) Semin. Haematol. 20, 225–242.
31 Chassis, J.A. and Mohandas, N. (1986) J. Cell Biol. 103, 343–350.
32 Cohen, C.M. (1983) Semin. Haematol. 20, 141–153.
33 Marchesi, V.T. (1983) Blood 61, 1–11.
34 Lux, S.E. and Palek, J. (1983) Semin. Haematol. 20, 189–224.
35 Liu, S.C., Palek, J., Prchal, J.T. and Castleberry, R.P. (1981) J. Clin. Invest. 68, 597–605.
36 Lawler, J., Liu, S.C., Palek, J. and Prchal, J. (1982) J. Clin. Invest. 70, 1019–1030.
37 Franck, P.F.H., Bevers, E.M., Lubin, B.H., Comfurins, P., Op den Kamp, J.A.F., Zwaal, R.F.A., Van Deenen, L.L.M. and Roelofsen, B. (1985) J. Clin. Invest. 75, 183–190.
38 Franck, P.F.H., Op den Kamp, J.A.F., Lubin, B., Berendsen, W., Joosten, P., Briet, E., Van Deenen, L.L.M. and Roelofsen, B. (1985) Biochim. Biophys. Acta 815, 259–267.
39 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2617.